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Original Article

Phytochemical, Free Radical Scavenging Activity and Thin Layer Chromatography Analysis of Methanolic Extracts of Six Wild Mushroom Species Collected From the Shai Hills Reserve of Ghana

Ebenezer Owusu[1*], Gladys Schwinger[1], Matilda Dzomeku[2], Mary Obodai[2] and Isaac Asante[1]

ABSTRACT

Objective: Six different mushroom species (*Termitomyces*, *Ganoderma*, *Amauroderma*, *Mycena*, *Marasmus* and an unknown) were studied to ascertain their phytochemical and antioxidant properties and determine their TLC analysis of methanolic extracts. **Materials and Methods:** The DPPH (1,1-diphenyl-2-picrylhydrazyl) model was employed to determine free radical scavenging activity of the methanolic extracts of the mushrooms, aluminum chloride calorimetric method for flavonoid, Thin layer chromatography (TLC) for retention factor and atomic absorption spectrophotometric (AAS) for macro and micronutrients. **Results:** Phytochemical analyses of the methanolic extract revealed the presence of antioxidants, phenols and flavonoids. The antioxidant values (IC_{50} (μg/ml)) ranged from 1.56 x 10^{-4} to 21.07 x10^{-4}. Total phenol content ranged between 2.54 and 17.53 mg/g GAE with a mean of about 11.27 mg/g GAE. Total flavonoid content also ranged from 5.46 to 23.75 mg/g RUE with a mean of 13.41 mg/g RUE. Micronutrients such as cadmium, iron, lead, manganese and zinc were determined. Also macronutrients determined included, calcium, potassium, magnesium and sodium. Sodium ranged the highest with values ranging 6966.67 mg/L to 9600.00 mg/L followed by iron ranging from 1613.67 to 3040.00 mg/L. Percentage crude protein ranged between 11.09 % and 28.24 %. Alkaloid was present in only *Mycena* sp and *Ganoderma* sp with a band each of R_f value of 0.49. A total of 18 different bands were recorded for flavonoid with R_f values that ranged between 0.20 and 0.97, respectively. **Conclusion:** The antioxidant potential of the methanolic extracts of the mushroom samples in this study recorded higher values. This indicates that mushrooms have high antioxidant properties and rationalizes further investigation in the potential discovery of new natural bioactive principles from these mushrooms.

Key words: Antioxidant, Extracts, Flavonoids, Mushrooms, Phytochemical.

Ebenezer Owusu[1*], Gladys Schwinger[1], Matilda Dzomeku[2], Mary Obodai[2] and Isaac Asante[1]

[1]Dept. of Plant and Environmental Biology, University of Ghana, Legon-Accra, GHANA.
[2]CSIR-Food Research Institute, P, O, Box, M20, Accra, GHANA.

Correspondence

Ebenezer Owusu

Department of Plant and Environmental Biology, University of Ghana, Legon-Accra, GHANA.

Phone no: 233-264-263531

E-mail: eowusu@ug.edu.gh

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INTRODUCTION

Mushrooms are low in calories and high in proteins, vitamins and minerals and they therefore serve as valuable, healthy foods.[1] Currently, the consumption of wild edible mushroom is increasing as a result of their good source of protein and micronutrients.[2] Apart from their healthy food source, mushrooms serve as therapeutic foods and are also useful in the management of such diseases as hypertension, hypercholesterolemia and cancer, diabetes, neurodegenerative disorders and cardiovascular disorders. Mushrooms are also good sources of antioxidants and are therefore possible protective agents for the reduction of oxidative damage in humans without any side effects.[3] In recent times edible mushrooms have attracted interest as functional foods as well as source of bioactive metabolites for the development of drugs and nutraceuticals.[4,5] Some have also been found to be a source of phenolic compounds,[6] flavonoids, terpenoids, sterols, ascorbic acid, ergothioneine and carotenoids.[3,4,5,6,7]

Various methods are available for the screening of pharmacologically active substances in extracts. One such method is the thin layer chromatography (TLC), a simple, quick reliable and inexpensive procedure that can be used for screening of plant extracts.[8] The TLC is also very useful for preliminary study before other instrumental techniques are applied.[9,10] The use of TLC for separation and purification of plant constituents depends on their size, shape and charge.[11] It serves as a quick way for monitoring the identity and purity of drugs; it is also used for detecting adulterations and substitutions, drug combination analysis and phytochemical preparations.[12,13]

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The aim of this study was to characterize mushroom species collected from Shai Hills Reserve in Ghana on the basis of their nutrient content, phytochemical constituents and antioxidant potentials.

MATERIALS AND METHODS

Sample collection

Mushroom samples were collected from the Shai Hills Reserve in Ghana in 2014. These were collected in brown large envelopes and identified using cultural and morphological characteristics.[14,15] The samples were later sun-dried and kept in the freezer until ready for use.

Preparation of sample

Mushroom samples were prepared by following a modified approach.[16] The mushrooms were lyophilized (Labconco, Missouri) and pulverized into fine powder. Ten grams of the pulverized samples was extracted with 100 ml of methanol at 25°C at 20 g for 24 hours and filtered through Whatman No. 1 filter paper. The residue was extracted with two additional 100 ml portions of methanol as described above and combined ethanolic extracts were concentrated under reduced pressure below 40°C to obtain the crude extract. The crude extracts were re-dissolved in methanol at concentration 20 mg/ml and stored at 4°C for further analyses.

Antioxidant Activity (DPPH Free Radical Scavenging Activity) of Methanolic Extract

The diluted working solutions of the test extracts were prepared in methanol. Accurately 100 µl of test samples (0.6-20.0 mg/ml) in methanol was added with 5 µl DPPH solution in 96-well microtitter plates. An amount of 0.002% DPPH was prepared in methanol. One microliter of this solution was mixed with 1 ml of sample solution and the standard solution to be tested separately. These solution mixtures were kept in the dark for 20 min and optical density was measured at 517 nm using a spectrophotometer against methanol. The blank was used as 1 ml of methanol with 1 ml of DPPH solution (0.002%). The optical density was recorded and percent of inhibition was calculated using the formula given below.

Percent inhibition of DPPH activity = (A–B/A) × 100 where A is optical density of the blank and B is optical density of the sample.

Statistics and IC_{50}

Decolorization was plotted against the sample extract concentration and a linear regression curve was established in order to calculate IC_{50} (µg/ml), which is the amount of sample required to decrease the absorbance of the DPPH free radical by 50%. All the analyses were carried out in triplicate and the results expressed as mean ± SD. Statistical analyses were performed using SAS computer software

Determination of total phenol content

Total phenolic content in the methanolic extracts was determined by using Folin-Ciocalteau reagent based on modified version.[17] Each sample (150 µl, 10 mg/ml) was added with 1200 µl distilled water and 450 µl aqueous sodium carbonate solution. One hundred microliters of Folin-Ciocalteu reagent was added to the mixture and agitated. The mixture was allowed to stand for 90 minutes and the absorbance was measured at 760 nm by using UV/visible spectrophotometer (SpectraMax Plus384, United States). The concentration of total phenolic compounds was calculated based on standard curve of gallic acid (0.2-1.0 mg/ml) with the linear equation, $y = 0.624x - 0.939$, where $R2 = 0.995$. The results were expressed as µg of gallic acid equivalent (GAE/ µg) per gram of the extracts.

Determination of total flavonoid content

The modified aluminum chloride colorimetric method[18] was used to determine flavonoid content. Mushroom extract (100 µl, 10 mg/ml) was mixed with distilled 500 µ water and sodium nitrite, $NaNO_2$ (5%, 30 µl). The mixture was allowed to stand for 5 minutes. Aluminium chloride solution, $AlCl_3.H_2O$ (10%, 60 µl) was added to the mixture and left for 6 minutes. Sodium hydroxide, NaOH (1M, 200 µl) and 110 µl distilled water were added to the solution and mixed well. Absorbance of the solution was measured at 510 nm (SpectraMax Plus384, United States) and the concentration of total flavonoids content was calculated based on standard curve of rutin (0.2-1.0 mg/ml) with the linear equation $y = 0.0101x + 0.2238$, where $R2 = 0.9563$. The results were expressed as µg of rutin equivalent (RE/ µg) per gram of the extracts.

Determination of microelements and microelements

The micro and macronutrients were determined by an atomic absorption spectrophotometric (AAS) method. The samples were digested in nitric acid solution and passed through the AAS system using different lamps. Calibration was done with related minerals in different concentrations for different mineral elements.

Determination of crude protein

An amount of 0.1 grams of the pulverized mushroom sample was weighed into labelled conical flasks and 5 ml of concentrated H_2SO_4 was added and shaken gently. The solution was placed on a preheated sand digester and after heating for a while, drops of H_2O_2 was added. The solution was made to cool after which it was topped with distilled water to 100 ml mark of the volumetric flask. An amount of five milliliters of the solution was transferred into a round bottom flask, after which five milliliters of 2% boric acid solution was added. The solution was distilled in the presence of 5 ml of 40% NaOH. The distillate was against 0.1M HCl until a colour change was observed. The titre value was recorded. The procedure was repeated three times for each sample to minimize error. Percent nitrogen was calculated by using the following formula:

$$\text{Nitrogen (\%)} = \frac{(a-b)\times 0.01\times 14\times V\times 100}{1000\times W\times \text{aliquot pipetted}}$$

Where *a* represents titre value of sample, *b*, the titre value of the blank, *V* represents the extraction volume (100 ml) and *W* represents the weight of the sample (0.1 g). The crude protein content was estimated by multiplying the percent nitrogen value by the factor 6.25.

Thin layer chromatography (TLC)

Methanolic extracts of mushroom samples were separated on silica gel thin layer aluminium plates of 15x5 cm with 3 mm thickness. Extracts were spotted manually using capillary tube. Solvent systems used for the separation of the following phytochemical compounds alkaloids, flavonoids, saponins and terpenes were a mixture of chloroform and diethylamine (9 : 1), a mixture of chloroform and ethylacetate (6 : 4), a mixture of chloroform : methanol : distilled water : toluene (8 : 1 : 0.5 : 0.5) and toluene, respectively. After separation of the phytochemicals compounds, reagents such as iodine and Dragendorff were used to identify the compounds. Colour of the spots was noted and retention factor (Rf) values calculated by using the following formula:

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}} \times 100$$

RESULTS AND DISCUSSION

Antioxidant potential

The extracts of *Ganoderma sp* showed the strongest activities for DPPH radicals and its IC_{50} value was 1.56 x x 10^{-4} µg/ml (Table 1). The extract for the unknown species gave the lowest activity. Moderate activity was recorded by the extracts of *Mycena* sp, *Marasmus* sp, *Termitomyces sp* and *Amauroderma* sp.

Radical scavenging activity of mushroom samples studied by Shirmila and Radhamany[3] was 450 µg/ml. The antioxidant potential of the methanolic extracts of the mushroom samples in this study recorded higher values.

Total phenol and flavonoid content

The highest total phenol content of 17.53 mg gallic acid equivalent g was recorded for *Mycena* sp. While the lowest amount was realised from the extract of *Amauroderma* sp (Table 1). Total phenol content as reported in this work is higher than those reported by Shirmila and Radhamany[3] who reported a total mean value of 1.901+0.011 mg GAE /g of extract.

The highest flavonoid content of 23.75 mg/g RUE was recorded by *Termitomyces* sp. whilst *Amauroderma* sp. gave the lowest amount (Table 1). Total flavonoid content reported in the current study is higher than the value reported by Shirmila and Radhamany[3]. They reported a value of 0.39 mg quercitin equivalent/g of extract. However the values for the current work were lower than those other researchers who reported 248+7.63 mg/g and 42.063 mg/g for total phenol and total flavonoid, respectively.[19]

Micronutrient contents of mushrooms

Amauroderma sp had the lowest content of cadmium (2.30 mg/L) while the highest content of 8.40 mg/L was recorded by the unknown sp. (Table 2).

The most abundant micronutrient was found to be iron ranging from 1613.67 mg/L to 3731.00 mg/L. Iron values in mushroom samples as reported[20] ranged from 568–3904 lg/g. The iron values of the present study fall within this range.

Lead was highest in extract of *Amauroderma* sp. while the lowest was recorded for *Marasmus* sp. Manganese was highest in *Marasmus* (222.00 mg/L) and lowest in *Ganoderma* sp (26.00 mg/L). Zinc was highest in the unknown sp. (81.90 mg/L) and lowest in *Amauroderma* sp. (36.97 mg/L). Zinc content of mushroom samples ranged from 33.5–89.5 lg/g.[23] Others[21,22] also reported values that ranged from 29.3–158 lg/g and 45–188 lg/g, respectively. There were significant differences ($P < 0.001$) among the six different mushroom species with respect to heavy metal content. The manganese content of the mushrooms studied in the present work ranged from 26 mg/L to 222.00 mg/L. However, others also reported values that ranged from 7.1-91.3 lg/g,[22] and values that ranged from 21.7–74.3 lg/g.[23]

Macronutrients and percentage crude protein content of mushrooms

Marasmus sp. recorded the highest amount of calcium (900.00 mg/L) while *Termitomyces* sp. scored the lowest (127.33 mg/L) (Table 3), however other researchers recorded lower values of 0.17 to 8.80 mg/g dw[1]. Potassium content was highest in the unknown sp. (5633.33 mg/L) and lowest in *Amauroderma* sp. (1000.00 mg). These values were higher than those recorded by Huseyin, *et al*. (2009) which ranged from 12.6-29.1 mg/g dw. The highest amount of magnesium was recorded by the unknown sp. (754.33 mg/L) while the lowest was recorded by *Termitomyces* sp. (214.67 mg/L). The level of magnesium content reported in this work was higher than those of other researchers who reported values that ranged from 0.90 mg/g to 4.54 mg/g dw. Sodium content was highest in the unknown sp. (9600.00 mg/L).[1]

Table 1: Free radical scavenging activity, total phenolic and total flavonoid mean content of the six mushroom species.

Mushroom species	DPPH scavenging activity IC_{50} (µg/mL) (x 10^{-4})	Total Phenol (mg/g extract) in GAE	Total flavonoid (mg/g extract) in RUE
Marasmus	5.6533	9.34100	11.23500
Unknown	21.0667	9.05200	12.81286
Amauroderma sp.	7.5200	2.54133	5.46498
Mycena sp.	4.6867	17.52800	9.33410
Termitomyces sp.	5.6000	16.80800	23.75048
Ganoderma sp.	1.5600	12.34233	17.87333
Mean	**7.68**	**11.26878**	**13.41179**
Lsd $_{(0.05)}$	**1.00**	**0.1959**	**0.0503**

Table 2: Mean content of micronutrients in six different mushroom species from the Shai Hills

	Cadmium	Iron	Lead	Manganese	Zinc
Marasmus	2.30	3731.00	99.00	222.00	62.30
Unknown	8.40	1816.67	155.33	61.33	81.90
Amauroderma sp.	8.00	3040.00	422.33	129.00	36.97
Mycena sp.	5.30	2207.33	120.00	70.00	70.17
Termitomyces sp.	7.33	1624.67	141.67	59.00	38.87
Ganoderma sp.	7.73	1613.67	411.33	26.00	65.33
Mean	**6.51**	**2338.89**	**224.94**	**94.56**	**59.26**
Lsd $_{(0.05)}$	**0.36**	**21.93**	**35.69**	**7.22**	**3.44**

Table 3: Mean content of macronutrient and percentage crude protein in six different mushroom species from the Shai Hills

	Calcium	Potassium	Magnesium	Sodium	Protein
Marasmus	900.33	2466.67	612.67	6966.67	20.54
Unknown	813.00	5633.33	754.33	9600.00	21.24
Amauroderma sp.	829.67	1000.00	440.67	7433.33	11.09
Mycena sp.	389.67	4133.33	632.67	9333.33	28.24
Termitomyces sp.	127.33	4900.00	214.67	9133.33	21.88
Ganoderma sp.	134.33	3166.67	439.00	8066.67	26.89
Mean	**532.39**	**3550.00**	**515.67**	**8422.22**	**21.64**
Lsd $_{(0.05)}$	**79.44**	**83.86**	**3.458**	**139.07**	**1.01**

Table 4: TLC fingerprint for alkaloids in methanolic extracts of six different mushroom species from the Shai Hills

Fractions	R_f values	*Marasmus*	Unknown	*Amauroderma* sp.	*Mycena* sp.	*Termitomyces* sp. (nkankuma)	*Ganoderma* sp.
	0.49	-	-	-	+	-	+

Table 5: TLC fingerprint for flavonoids in methanolic extracts of six different mushroom species from the Shai Hills

Fractions	R_f values	*Marasmus*	Unknown	*Amauroderma* sp.	*Mycena* sp.	*Termitomyces* sp. (nkankuma)	*Ganoderma* sp.
A	0.20	+	-	-	-	-	-
B	0.21	-	-	-	-	-	+
C	0.22	-	+	-	-	-	-
D	0.23	-	-	-	+	-	-
EF	0.25	-	-	-	-	+	-
G	0.40	-	-	+	-	-	-
H	0.43	-	+	-	-	-	-
I	0.51	-	-	-	-	+	-
J	0.63	-	+	-	-	+	+
K	0.70	-	-	-	-	-	+
L	0.72	+	-	-	-	-	-
M	0.73	-	-	-	+	-	-
N	0.76	-	-	-	-	-	+
O	0.87	-	-	-	-	+	-
P	0.88	-	+	-	-	-	-
Q	0.90	-	-	-	-	-	+
R	0.96	-	-	-	-	+	-
S	0.97	-	+	-	-	-	-
Total number of bands		**2**	**5**	**1**	**2**	**5**	**5**

The highest percentage of crude protein was recorded by *Mycena* sp. (28.24 %) and the lowest was recorded by *Amauroderma* sp. (11.09 %). There were significant differences ($P < 0.001$) among the six mushroom species with reference to the major elements and protein contents (Table 3). In a proximate and mineral composition analysis of four edible mushrooms in Nigeria, researchers reported 37% crude protein in *Termitomyces mammiformis*.[24]

TLC profile for alkaloid

Alkaloid was present in only *Mycena* sp and *Ganoderma* sp with a band each of R_f value of 0.49 (Table 4).

TLC profile for flavonoid

A total of 18 different bands were recorded with R_f values that ranged between 0.20 and 0.97 (Table 5). Two different bands were recorded by the *Marasmus* sp. with R_f values of 0.2 and 0.72, respectively. Five different bands were recorded by the unknown sp. with R_f values of 0.22, 0.43, 0.63 and 0.88, respectively. *Amauroderma* sp. recorded only one band with R_f value of 0.40. *Mycena* sp. recorded a total of two different bands of R_f values of 0.23 and 0.73, respectively. A total of five different bands were recorded by *Termitomyces* sp. with R_f values of 0.25, 0.51, 0.63, 0.97 and 0.97, respectively. The *Ganoderma* sp. also recorded a total of five

Table 6: TLC fingerprint for saponins in methanolic extracts of six different mushroom species from the Shai Hills

Fractions	R_f values	*Marasmus*	Unknown	*Amauroderma* sp.	*Mycena* sp.	*Termitomyces* sp. (nkankuma)	*Ganoderma* sp.
A	0.16	-	+	-	-	-	-
B	0.41	-	+	-	-	-	-
C	0.44	-	-	-	-	+	-
D	0.59	+	-	-	-	-	-
E	0.62	-	-	-	-	-	+
F	0.66	+	-	-	-	-	-
G	0.68	-	-	-	+	-	+
H	0.69	-	-	-	+	-	-
I	0.71	-	-	+	-	-	-
J	0.73	-	+	-	-	-	-
K	0.78	-	-	+	-	-	-
L	0.79	-	+	-	-	-	-
M	0.88	-	-	-	-	+	-
N	0.94	+	-	-	+	-	+
O	0.99	+	-	-	-	-	+
Total number of bands		**4**	**4**	**2**	**3**	**2**	**4**

Table 7: TLC fingerprint for terpenes in methanolic extracts of six different mushroom species from the Shai Hills

Fractions	R_f values	*Marasmus*	Unknown	*Amauroderma* sp.	*Mycena* sp.	*Termitomyces* sp.	*Ganoderma* sp.
A	0.07	-	+	+	-	-	-
B	0.10	+	-	-	-	-	-
C	0.11	-	-	-	+	-	-
D	0.13	-	-	-	-	-	+
E	0.16	-	-	-	-	+	-
F	0.27	-	+	-	-	-	-
G	0.28	-	-	-	-	+	-
H	0.31	-	-	-	-	-	+
I	0.37	-	-	+	-	-	-
J	0.43	-	-	-	-	-	+
K	0.55	-	-	-	-	+	-
L	0.56	-	+	+	-	-	-
M	0.61	+	-	-	+	-	+
Total number of bands		**2**	**3**	**3**	**2**	**3**	**4**

different bands of R_f values of 0.21, 0.63, 0.70, 0.76 and 0.90, respectively. The band with R_f value of 0.63 was common to the unknown species, *Termitomyces* sp. and the *Ganoderma* sp.

TLC profile for saponin

A total of 15 different bands were recorded with R_f values ranging from 0.16 to 0.99 (Table 6). Extracts of *Marasmus* sp. recorded a total of four different bands with R_f values of 0.59, 0.66, 0.94 and 0.99, respectively. A total of four different bands were recorded by extracts from the unknown sp. at R_f values of 0.16, 0.41, 0.73 and 0.79, respectively. Extracts of the *Amauroderma* sp. gave two bands with R_f values of 0.71 and 0.78, respectively. A total of three different bands were recorded by extracts from the *Mycena* sp. with R_f values of 0.68, 0.69 and 0.94, respectively. Extracts of the *Termitomyces* sp. gave a total of two different bands with R_f values of 0.44 and 0.88, respectively. The *Ganoderma* sp. extracts gave a total of four different bands with R_f values of 0.62, 0.68, 0.94 and 0.99, respectively. The band with R_f value of 0.68 was common to the *Mycena* and *Ganoderma* spp. The band with the R_f value of 0.84 was common to the *Marasmus, Mycena* and *Ganoderma* spp. Similarly the band with the R_f value of 0.99 was common to the *Marasmus* and *Ganoderma* spp.

TLC profile for terpene

A total of 13 different bands were scored with R_f values ranging between 0.07 and 0.61 (Table 7). Extracts from the Marasmus sp. recorded a total of 2 different bands with R_f values of 0.10 and 0.61, respectively. Extracts from the unknown sp. recorded a total of three different bands of R_f values

of 0.07, 0.27 and 0.56, respectively. The *Amauroderma* sp. extracts gave a total of three different bands with R_f values of 0.07, 0.37 and 0.56, respectively. Extracts from the *Mycena* sp. gave a total of 2 bands with R_f values of 0.11 and 0.61, respectively. The extracts from *Termitomyces* sp recorded a total of three different bands with R_f values of 0.16, 0.28 and 0.55, respectively. The *Ganoderma* sp. extracts gave a total of four different bands with R_f values of 0.13, 0.31, 0.43 and 0.61, respectively. The bands with R_f value of 0.07 and 0.56 was common to the unknown and *Amauroderma* spp. Similarly, the band with R_f value 0.61 was common to the *Mycena* and *Ganoderma* sp.

TLC profiles of mushrooms is a new area of research in Ghana. The TLC revealed the presence of single alkaloid compound which was present in only *Mycena*. The highest number of flavonoid compounds was detected in extracts of *Ganoderma* sp, *Termitomyces* and the unknown sp. concerning saponin profile the highest number of compounds were present in extracts of *Marasmus* sp. *Ganoderma* and the unknown sp. The highest number of terpene compounds was detected in extracts of *Ganoderma* sp.

CONCLUSION

The antioxidant potential of the methanolic extracts of the mushroom samples in this study recorded higher values. This indicates that mushrooms have high antioxidant properties and rationalizes further investigation in the potential discovery of new natural bioactive principles from these mushrooms.

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CONFLICT OF INTEREST

None

ABBREVIATIONS USED

AAS: Atomic absorption spectrophotometric**; DPPH**: 1, 1-diphenyl-2-picrylhydrazyl**; IC_{50}**: half maximal inhibitory concentration**; GAE**: Gallic acid equivalent**; R_f**: Representative Fraction**; RUE**: Rutin equivalent**; TLC**: Thin layer chromatography.

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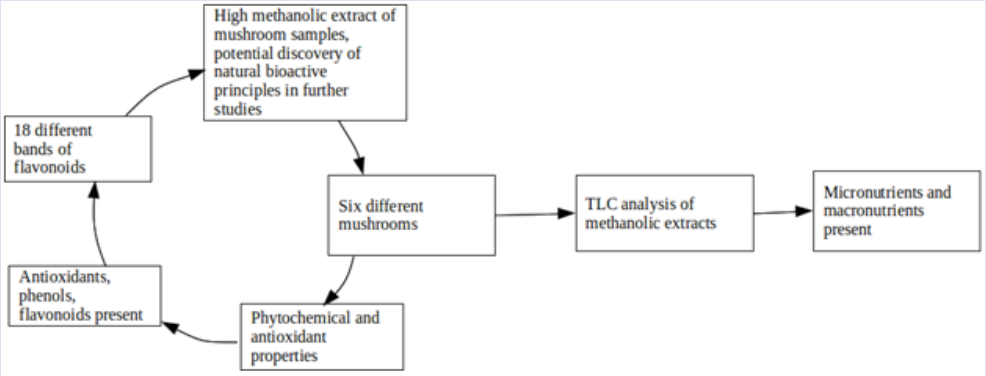
GRAPHICAL ABSTRACT



SUMMARY

- Phytochemical analyses of the methanolic extract of the mushrooms revealed the presence of antioxidants, phenols and flavonoids.
- The highest number of flavonoid compounds was detected in extracts of *Ganoderma* sp, *Termitomyces* and the unknown sp.
- There is the need for further investigation with the view of discovering new natural bioactive principles from these mushrooms.
- TLC profiles of mushrooms is a new area of research in Ghana.

ABOUT AUTHORS

Dr. Ebenezer Owusu is a Senior Lecturer at the Department of Plant and Environmental Biology at the University of Ghana. He is a member of many scientific associations and has publications in many peer-reviewed journals.

Mrs. Gladys Schwinger was an Assistant Lecturer at the Department of Plant and Environmental Biology at the University of Ghana. She is currently pursuing a PhD degree at the Institute for Environmental and Sanitation Studies (IESS), University of Ghana.

Matilda Dzomeku is a Research Scientist and the head of the mushroom laboratory of CSIR-Food Research Institute.

Professor Mary Obodai is a Principal Research Scientist and the Director of CSIR-Food Research Institute. She is an Associate Professor at the CSIR college of Science and Technology.

Professor I. K. Asante is professor and Head of the Department of Plant and Environmental Biology at the University of Ghana. He has published extensively in many peer-reviewed journals.

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